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(57) Abstract

The present invention provides methods of generating recombinant human antibodies directed against cancer associated tumor antigens by isolating peripheral blood lymphocytes from tumor bearing patients, detecting or identifying B–cell clones producing antibodies from theses lymphocytes, isolating cDNA encoding immunoglobulins from such B–cell clones, expressing the immunoglobulin encoded by the cDNA and isolating the immunoglobulin as a functional antibody.

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METHODS FOR PRODUCING HUMAN TUMOR ANTIGEN SPECIFIC ANTIBODIES

Technical Field

The claimed invention relates generally to the detection and isolation of B lymphocyte cells that secrete tumor antigen specific antibodies, as well as to the purification of such antibodies and the genes that encode these antibodies. The invention further relates to compositions comprising tumor antigens fused to immunoglobulin Fc domains as well as methods of using the compositions to detect tumor antigen specific antibodies and the B cells which secrete these antibodies.

Background of the Invention

It is fairly well established that the phenotypic changes which distinguish a neoplastic cell from its preneoplastic (or normal) analogues can be the result of one or more changes in the expression of specific gene products, including the loss of normal cell surface components or the gain of others (*i.e.*, antigens not detectable in corresponding normal, non-neoplastic tissue). The genes which are expressed in neoplastic or tumor cells, but not in normal cells, or which are expressed in neoplastic cells at levels substantially above those found in normal cells, have been termed tumor specific genes and their gene products, "neoantigens." Such neoantigens may serve as markers for tumor phenotype (Roitt, *et al.*, in Immunology (2nd ed), pp 18.1-18.18, Gower Medical Publishing, New York, NY (1989)).

Early research on murine tumors revealed that certain tumor specific gene products, when displayed on the tumor cell membranes, led to the rejection of tumor cells when they were transplanted into syngeneic animals (see Prehn, *et al.*, *J Natl Canc Inst* 18: 769-778 (1957); Klein, et al., *Cancer Res* 30: 1560-1572 (1960); and Gross, *Cancer Res* 3: 326-333 (1943)). Such tumor specific antigens are recognized by T-cells in the recipient animal and provoke a cytolytic T-cell response with accompanying lysis

of the transplanted cells. During the last few years, significant progress has been made in the identification of tumor associated antigens recognized by cytotoxic T lymphocytes (see Kirkin, *et al.*, *APMIS* 106: 665-679 (1998); Stockert, *et al.*, *J Exp Med* 187: 1349-1354 (1998); Tureci, *et al.*, *Int J Cancer* 77: 19-23 (1998)).

In contrast to the use of cytoxic T cells in the treatment of cancer, it recently was discovered that cancers may express various antigens against which a cancer patient makes detectable antibodies. Thus, the technique called "serological analysis of recombinant cDNA expression libraries" (SEREX) using mRNA extracted from tumor cells in conjunction with autologous patient serum provides a powerful approach to identify immunogenic tumor antigens (see Chen, *et al.*, *Proc Natl Acad Sci USA* 94: 1914-1918 (1997)). Exploitation of such methods has led to the assignment of these antigens to three main groups: cancer/testis-specific antigen (MAGE, BAGE, GAGE, PRAME and NY-ESO-1, melanocyte differentiation antigens (tyrosinase, Melan-A/MART, gp100, TRP-1 and TRP-2) and mutated or aberrantly expressed antigens (MUM-1, CDK4, beta-catenin, gp100-in4, p15 and N-acetylglucosaminyltransferase V). See Kirkin, et al., *APMIS* 106: 665-679 (1998). These antigen molecules may serve as reagents which can be used to generate diagnostic and/or therapeutic agents. One such use is to induce the production of antibodies specific to a particular tumor antigen marker using conventional immunization and antibody production techniques.

The use of human antibodies in the detection and treatment of human cancer has been limited by the apparent scarcity of antibodies to tumor cell antigens (see Haspel, *et al.*, *Cancer Res* 45: 3951-3961 (1985)). This may be due in part to the existence of tolerance to and the genuinely low immunogenicity of these "self" antigens (see Kirkin, *et al., APMIS* 106: 665-679 (1998); and Kamigaki, *et al., Cancer Res* 85: 298-305 (1994)). Attempts to obtain therapeutic or diagnostic reagents has led to the development of chimeric human-murine antibodies, wherein different anti-neoantigen fusion antibodies are generated recombinantly (Kamigaki, *et al.*, *Cancer Res* 85: 298-305 (1994); Sha, *et al.*, *Cancer Biother* 9: 341-349 (1994); Beidler, et al., *J Immunol* 141: 4053-4060 (1988); Sahagan, et al. *J Immunol* 137:1066-1074 (1986); Qi, *et al.*, *J Surg*

Oncol 59: 3-9 (1995); and Steffens, *et al., J Clin Oncol* 15:1529-1537 (1997)). However, while these molecules often show identical specificity to human tumor antigen epitopes, problems exist in that various degrees of immunogenicity for these antigens are observed, and therefore significant investments of labor and time are required, for example, for the construction of appropriate structural motifs to make a functional immunogenic fusion protein.

Summary of the Invention

The present invention provides methods of generating recombinant human antibodies directed against cancer associated tumor antigens comprising the steps of: a) isolating peripheral blood lymphocytes from tumor bearing patients; b) detecting or identifying B-cell clones producing antibodies from these lymphocytes; c) isolating cDNA encoding immunoglobulins from such B-cell clones; d) expressing the immunoglobulin encoded by the cDNA of step (c); and e) isolating the immunoglobulin as a functional antibody.

The present invention also includes methods of detecting and isolating human B cell clones which produce tumor antigen specific antibodies.

General Description

The term "antibody" as used herein, unless indicated otherwise, is used broadly to refer to both antibody molecules and a variety of antibody derived molecules. Such antibody derived molecules comprise at least one variable region (either a heavy chain of light chain variable region) and include molecules such as Fab fragments, Fab' fragments, F(ab')2 fragments, Fd fragments, Fabc fragments, Sc antibodies (single chain antibodies), diabodies, individual antibody light chains, individual antibody heavy chains, chimeric fusions between antibody chains and other molecules, and the like.

Antibodies described herein may contain alterations of the amino acid sequence compared to a naturally occurring antibody. In other words, the framework regions of some of the antibodies of the invention do not necessarily consist of the precise amino

acid sequence of the framework region of a natural occurring human antibody variable region, but contain various substitutions that improve the binding properties of the antibody to its cognate antigen or change the binding of the antibody to effector molecules such as complement or the Fc receptor. In another format, a minimal number of substitutions are made to the framework region in order to ensure minimal immunogenicity of the antibody in humans. In preferred embodiments of recombinant antibodies of the invention, any non-human framework regions used may be altered with a minimal number of substitutions to the framework region in order to avoid large-scale introductions of non-human framework residues.

The term "conventional molecular biology methods" refers to techniques for manipulating polynucleotides that are well known to the person of ordinary skill in the art of molecular biology. Examples of such well known techniques can be found in Molecular Cloning: A Laboratory Manual 2nd Edition, Sambrook et al, Cold Spring Harbor, N.Y. (1989). Examples of conventional molecular biology techniques include, but are not limited to, *in vitro* ligation, restriction endonuclease digestion, PCR, cellular transformation, hybridization, electrophoresis, DNA sequencing, cell culture, and the like.

The term "variable region" as used herein in reference to immunoglobulin molecules has the ordinary meaning given to the term by the person of ordinary skill in the art of immunology. Both antibody heavy chains and antibody light chains may be divided into a "variable region" and a "constant region". The point of division between a variable region and a contrast region may readily be determined by the person of ordinary skill in the art by reference to standard texts describing antibody structure, e.g., Kabat et al "Sequences of Proteins of Immunological Interest: 5th Edition" U.S. Department of Health and Human Services, U.S. Government Printing Office (1991).

Production of antigen fusion proteins.

Fusion proteins may be prepared comprising a fusion between any antigen, preferably a tumor antigen, and an IgG constant region, preferably a murine Fc region,

using conventional molecular biology methods. In one embodiment, the pSecTagA expression vector may be used for mammalian expression of the fusion proteins. In a first step, a vector containing the cDNA for murine IgG heavy chain constant region is generated. A cDNA for a murine IgG1 heavy chain constant region fragment IgG1-Fc may be cloned from any murine hybridoma cell clone which produces a IgG1 monoclonal antibody by PCR amplification of the appropriate fragment. The IgG1-Fc fragment may then be cloned into any appropriate expression vector which allows replication and expression of the resulting fusion protein. This plasmid may then be used as a basis for ligation of the tumor antigen cDNA fragment of interest upstream of the IgG-Fc cDNA to allow the expression of fusion proteins. When using a pSecTagA vector, resulting fusion proteins contain an antigen part, an IgG-Fc part, a cmyc-tail and 6xhis (allowing simple purification and detection).

As an added benefit, the generated constructs may be transferred into other expression vectors. For example, for expression in yeast, the *Pichia* vector pPicZalpha (Invitrogen) may be used. For expression in insect cells, the Baculovirus expression vector pMelBac (Invitrogen) may be used.

The identity of the tumor antigen entity to be fused to the IgG Fc region may be variable depending on the specific antigen, the degree of characterization of the antigen, the localization of immunologically relevant epitopes, etc. For instance, full length antigen cDNAs may be used to produce a fusion protein comprising the full length tumor antigen fused in frame to the Fc portion. In another embodiment, cDNA fragments encoding immunologically relevant portions of the tumor antigen may be used to produce fusion proteins, *e.g.*, particular B cell epitopes.

Isolation of human antigen specific B cells.

Antigen specific B cells may be isolated from convenient samples, such as peripheral blood lymphocytes from a cancer patient, by techniques known and available in the art. For instance, fusion proteins of the invention may be used to detect and isolate

B cells which express immunoglobulin which specifically binds to the tumor antigen portion of the fusion protein by affinity chromatography, fluorescent activated cell sorting (FACS) and other commonly used techniques such as Zn-chelating sepharose or protein-A sepharose (see Harlow *et al.*, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988).

As another example, tumor draining lymph nodes obtained from a cancer patient may be cut into fine pieces and meshed through a wire gauze using a rubber policeman. Pure B cells may be isolated using CD19 coated immunomagnetic beads. Antigen specific B cells may be isolated using the appropriate fusion protein by affinity chromatography or fluorescent activated cell sorting. The resulting tumor antigen specific B cells may then be immortalized using known techniques such as immortalization by EBV. Any effective lymphotropic virus or other transforming agent able to transform the B-cells to grow in continuous culture and still produce monoclonal antibodies specific for tumor associated antigens can be used.

Isolation of antigen specific immunoglobulin heavy and light chain sequences.

In addition to providing human tumor specific antibodies, the subject invention provides for polynucleotides encoding human tumor specific antibodies. The polynucleotides may have a wide variety of sequences because of the degeneracy of the genetic code. A person of ordinary skill in the art may readily change a given polynucleotide sequence encoding a tumor specific antibody into a different polynucleotide encoding the same human tumor specific antibody. The polynucleotide sequence encoding the antibody may be varied to take into account factors affecting expression such as codon frequency, RNA secondary structure, and the like.

Production of recombinant human antibodies

The antibodies of the subject invention may be produced by a variety of methods useful for the production of polypeptides, *e.g.*, in vitro synthesis, recombinant DNA

production, and the like. Preferably, humanized antibodies are produced by recombinant DNA technology.

The antigen specific antibodies of the invention may be produced using recombinant immunoglobulin expression technology. The recombinant production of immunoglobulin molecules, including humanized antibodies is described in U.S. Pat. No. 4,816,397 (Boss et al), U.S. Pat. No. 4,816,567 (Cabilly et al), U.K. patent GB 2,188,638 (Winter et al), and U.K. patent GB 2,209,757. Techniques for the recombinant expression of immunoglobulins, including humanized immunoglobulins, can also be found, among in Goeddel *et al.*, Gene Expression Technology Methods in Enzymology Vol. 185 Academic Press (1991), and Borreback, Antibody Engineering, W. H. Freeman (1992). Additional information concerning the generation, design and expression of recombinant antibodies can be found in Mayforth, Designing Antibodies, Academic Press, San Diego (1993).

As an example, the recombinant antibodies of the invention may be produced by the following process:

a) constructing, by conventional molecular biology methods, an expression vector comprising a nucleotide sequence that encodes an antibody heavy chain in which the CDRs and a minimal portion of the variable region framework that are required to retain donor antibody binding specificity are derived from the human immunoglobulin, and the remainder of the antibody is derived from another human immunoglobulin, thereby producing a vector for the expression of a humanized antibody heavy chain;

b) constructing, by conventional molecular biology methods, an expression vector comprising a nucleotide sequence that encodes an antibody light chain in which the CDRs and a minimal portion of the variable region framework that are required to retain donor antibody binding specificity are derived from the human immunoglobulin, and the remainder of the antibody is derived from another human immunoglobulin, thereby producing a vector for the expression of humanized antibody light chain;

c) transferring the expression vectors to a host cell by conventional molecular biology methods to produce a transfected host cell; and

d) culturing the transfected cell by conventional cell culture techniques so as to produce recombinant antibodies.

Host cells may be cotransfected with two expression vectors of the invention, the first vector encoding a heavy chain derived polypeptide and the second encoding a light chain derived polypeptide. The two vectors may contain different selectable markers but, with the exception of the heavy and light chain coding sequences, are preferably identical. This procedure provides for equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used which encodes both heavy and light chain polypeptides. The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA or both.

The host cell used to express the recombinant antibody of the invention may be a bacterial cell such as *Escherichia coli,* or antigen binding fragments may be expressed in available phage display systems (see Winter *et al.* (1994) Ann. Rev. Immunol., 12:433-455 and Little *et al.* (1995) J. Biotechnol., 41(2-3):187-195). Preferably a eukaryotic cell or most preferably a mammalian cell, such as a Chinese hamster ovary cell, may be used. The choice of expression vector is dependent upon the choice of host cell, and may be selected by a person skilled in the art so as to have the desired expression and regulatory characteristics in the selected host cell.

The general methods for construction of the vector of the invention, transfection of cells to produce the host cell of the invention, culture of cells to produce the antibody of the invention are all conventional molecular biology methods. Likewise, once produced, the recombinant antibodies of the invention may be purified by standard procedures of the art, including cross-flow filtration, ammonium sulphate precipitation, affinity column chromatography, gel electrophoresis and the like.

Preparation of therapeutic or prophylactic compositions

The antibodies of the present invention may be used in conjunction with, or attached to other antibodies (or parts thereof) such as human or humanized monoclonal antibodies. These other antibodies may be reactive with other markers (epitopes)

characteristic for the disease against which the antibodies of the invention are directed or may have different specificities chosen, for example, to recruit molecules or cells of the human immune system to the diseased cells. The antibodies of the invention (or parts thereof) may be administered with such antibodies (or parts thereof) as separately administered compositions or as a single composition with the two agents linked by conventional chemical or by molecular biological methods. Additionally the diagnostic and therapeutic value of the antibodies of the invention may be augmented by labeling the humanized antibodies with labels that produce a detectable signal (either *in vitro* or *in vivo*) or with a label having a therapeutic property. Some labels, e.g. radionuclides may produce a detectable signal and have a therapeutic property. Examples of radionuclide labels include ^{125}I and ^{131}I. Examples of other detectable labels include a fluorescent chromophore such as fluorescein, phycobiliprotein ortetraethyl rhodamine for fluorescence microscopy, an enzyme which produces a fluorescent or colored product for detection by fluorescence, absorbance, visible color or agglutination, which produces an electron dense product for demonstration by electron microscopy; or an electron dense molecule such as ferritin, peroxidase or gold beads for direct or indirect electron microscopic visualization. Labels having therapeutic properties include drugs for the treatment of cancer, such as methotrexate and the like.

The subject invention also provides for a variety of methods for treating and/or detecting cancer cells. These methods involve the administration to a cancer patient of tumor specific antibodies, either labeled or unlabeled. One method of detecting cancer cells in a human involves the step of administering a labeled tumor specific antibody (labeled with a detectable label) to a human and subsequently detecting bound labeled antibody by the presence of the label. Alternatively, the tumor specific antibodies may be linked or conjugated to a therapeutic molecule such as ricin or other toxins.

The recombinant antibodies of this invention may also be used for the selection and/or isolation of human monoclonal antibodies, and the design and synthesis of peptide or non-peptide compounds (mimetics) which would be useful for the same diagnostic

and therapeutic applications as the antibodies (e.g. Saragovi et al., (1991) Science 253:792-795).

When the tumor specific antibodies of the invention are used *in vivo*, the antibodies are typically administered in a composition comprising a pharmaceutical carrier. A pharmaceutical carrier can be any compatible, non-toxic substance suitable for delivery of the monoclonal antibodies to the patient. Sterile water, alcohol, fats, waxes, and inert solids may be included in the carrier. Pharmaceutically accepted buffering agents or dispersing agents may also be incorporated into the pharmaceutical composition.

The antibody compositions of the invention may be administered to a patient in a variety of ways. Preferably, the compositions may be administered parenterally, i.e., subcutaneously, intramuscularly or intravenously. Thus, this invention provides compositions for parenteral administration which comprise a solution of the human antibody or a cocktail thereof dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine and the like. These solutions are sterile and generally free of particulate matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, etc. The concentration of antibody in these formulations can vary widely, e.g., from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

Actual methods for preparing parenterally administrable compositions and adjustments necessary for administration to subjects will be known or apparent to those skilled in the art and are described in more detail in, for example, Remington's

Pharmaceutical Science, 15th Ed., Mack Publishing Company, Easton, Pa. (1980), which is incorporated herein by reference.

Examples

Example 1 Preparation of Fc fusion vector

Plasmids were prepared for the expression of a secreted chimeric fusion protein consisting of an N-terminal SEREX antigen and C-terminal murine IgG heavy chain constant region. The pSecTagA plasmid (Invitrogen) was used in a two step cloning technique for the generation of new expression vectors.

Generation of pSecTagA-mIgG1-Fc: The pSecTagA-mIgG1-Fc vector was generated by ligation of an amplified murine IgG1 constant region cDNA fragment into the pSecTagA plasmid. mRNA was extracted from the murine hybridoma cell clone producing an IgG1 monoclonal antibody using standard techniques. First strand cDNA was synthesized using dT(18) primed mRNA and reverse transcriptase (Superscript, Gibco) with supplied buffers and conditions. Synthesis was performed at 42°C for 45 min. This first strand cDNA was used to amplify a IgG1 constant region cDNA fragment using polymerase chain reaction. Amplification was performed with oligonucleotides containing an *Xho1* restriction site.

The following oligonucleotides were used:

mIgG1-S CTC GAG gtg gac aag aaa att gtg ccc agg; and

mIgG1-AS CTC GAG aga gtg gga gag gct ctt ctc agt atg.

Amplification was performed for 30 cycles with annealing at 64°C for 1 min, extension at 72°C for 2 min, denaturation 94°C for 1 min followed by a final extension at 72°C for 8 min. The 699 bp amplified fragment was cloned into a TA- Overhang vector (TA cloning kit, Invitrogen). The integrity of the sequence was verified by sequencing with vector specific oligonucleotides using standard techniques. After sequence verification, the murine IgG1 cDNA fragment was cut from the TA vector using *Xho1* digestion and was ligated into *Xho1* digested, dephosphorylated pSecTagA vector. Correct cloning of the pSecTagA-mIgGFc resulting plasmid was also verified by

sequencing. This new generated vector was used for the cloning of fusion vectors in the Examples below.

pSecTagA-SCP1-mFc, pSecTagA-NYESO1-mFc, pSecTagA-Tyrosinase-mFc, pSecTagA-MAGE3-mFc, pSecTagA-cErbB2-Fc, pSecTagA-HERVK10gag-mFc were generated from pSecTagA-mIgGFc. In a second step, the different cDNAs encoding tumor antigens were cloned into the pSecTag-mIgG1Fc vector resulting in vectors encoding dimeric antigen-IgG fusion proteins. For this purpose, cDNAs for the various tumor antigens were amplified using specific oligonucleotides from human first-strand cDNA derived from testis (for SCP1, NYESO1, MAGE3), melanoma (tyrosinase), breast cancer (cErbB2) or seminoma (HERV-K10 gag). Amplification of cDNAs were performed with oligonucleotides containing restriction enzyme digestion sites. Amplified cDNAs were cloned into TA-Overhang vectors (TA cloning kit Invitrogen). The integrity of sequences were verified by sequencing with vector specific oligonucleotides using standard techniques. After sequence verification the cDNAs were cut out from the TA-vectors and were ligated in pSecTagA-mIgG-Fc vector.

Example 2 Preparation of SCP1 fusions.

Cloning of SCP1-cDNA fragments containing *EcoR1* overhangs: Amplification with specific oligonucleotides SCP1-S1 and SCP1-AS1 (both containing *EcoR1* sites) was used to obtain the SCP1 coding sequence (see U.S. Patent Application 08/892,702). Amplification was performed using testis cDNA for 35 cycles with annealing at 64°C for 1 min, extension at 72°C for 2 min, denaturation 94°C for 1 min followed by a final extension at 72°C for 8 min.

The following oligonucleotides were used:

SCP1-S1 a gga att ctc caa gcc aga gag aaa gaa gta cat; and

SCP1-AS1 ag gaa ttc tga gtc tct ttc ttc aat gat ctt atc ata.

The amplified fragment was then cloned into TA-Overhang vector (TA cloning kit, Invitrogen). After determining identity the insert was cut with *EcoR1* and ligated into pSecTagA-mIgGFc precut with *EcoR1* resulting in pSecTagA-SCP1A-mIgGFc.

Example 3 Preparation of NY-ESO fusion

Cloning of NY-ESO-1 cDNA fragment containing *EcoR1* sites: Amplification was done with specific oligonucleotides NYESO1-S and NYESO1-AS (both containing *EcoR1* sites) (see U.S. Patent 5,804,381). Amplification was performed using testis cDNA for 35 cycles with annealing at 60°C for 1 min, extension at 72°C for 2 min, denaturation 94°C for 1 min followed by a final extension at 72°C for 8 min. The amplified cDNA fragment was cloned into a TA-overhang vector (TA cloning kit from Invitrogen).

The following oligonucleotides were used:

NY-ESO1-S-EcoR1 gaa ttc cca tgc agg ccg aag aag gcc; and

NY-ESO1-AS gaa ttc ctg cag ttg gcg gtg gtc.

After determining identity, the insert was cut with *EcoR1* and ligated into pSecTagB-mIgGFc precut with *EcoR1* resulting in pSecTagA-NY-Eso1-mIgGFc.

Example 4 Preparation of Tyrosinase fusions

Cloning of Tyrosinase cDNA fragment containing *BamH1* and *EcoR1* sites: Tyrosinase cDNA was amplified with specific oligonucleotides Tyrosinase-S, (containing a *BamH1* site) and Tyrosinase-AS (containing an *EcoR1* site) (the sequence of the tyrosinase cDNA is available in GenBank). Amplification was performed using melanoma derived first strand cDNA for 35 cycles with annealing at 64°C for 1 min, extension at 72°C for 2 min, denaturation 94°C for 1 min followed by a final extension at 72°C for 8 min. The amplified fragment was cloned into TA-Overhang vector (TA cloning kit, Invitrogen).

The following oligonucleotides were used:

Tyrosinase-S (*BamH1*) ag gat cca cat ttc cct aga gcc tg gtc tcc tct; and

Tyrosinase-AS (*EcoR1*) a gaa ttc tag ata gct ata gtc ata gcc cag atc.

After determining identity, the insert was cut with *BamH1* and *EcoR1* and ligated into pSecTagA-mIgGFc precut with *BamH1/EcoR1* resulting in pSecTagA-tyrosinase-mIgGFc.

Example 5 Preparation of cErbB2 fusions

Cloning of cErbB2: cErbB2(HER2/neu) cDNA was amplified with specific oligonucleotides cErbB2-S, (containing *Sfi1* and *Sal1* sites) and cErbB2-AS (containing a *Mun1* site) (the sequence of cErbB2 is available in GenBank). Amplification was performed for 35 cycles with annealing at 64°C for 1 min, extension at 72°C for 3 min, denaturation 94°C for 1 min followed by a final extension at 72°C for 8 min.

The following oligonucleotides were used:

cErbB2-S ag gcc cag ccg gcc atg tcg aca agc acc caa gtg tgc acc ggc aca gac; and

cErbB2-AS caa ttg gat gga cgt cag agg gct ggc tct atg.

The amplified fragment was cloned into a TA-Overhang vector (TA cloning kit Invitrogen). After determining identity, the insert was cut with *Sfi1* and *Mun1* and ligated into pSecTagA-mIgGFc precut with Sfi1/*EcoR1* resulting in pSecTagA-cErbB2-mIgGFc.

Example 6 Preparation of MAGE -3 fusions

Cloning of MAGE-3 cDNA fragment containing *Kpn1* sites: Amplification was done with specific oligonucleotides MAGE3-S and MAGE3-AS (both containing *Kpn1* sites) (see WO92/20356). Amplification was performed using MAGE3-plasmid provided from Pierre van der Bruggen (LICR, Brussel) for 20 cycles with annealing at 60°C for 1 min, extension at 72°C for 2 min, denaturation 94°C for 1 min followed by a final extension at 72°C for 8 min.

The following oligonucleotides were used:

MAGE3-Kpn1-S-acc aga gtc atg gta cct ctt gag cag agg agt; and

MAGE3-Kpn1-AS ggt acc ctc tct tcc ccc tct ctc aaa acc cac tc.

The amplified fragment was cloned into a TA-Overhang vector (TA cloning kit Invitrogen). After determining identity the insert was cut with *Kpn1* and ligated into pSecTagA-mIgGFc precut with *Kpn1* resulting in pSecTagA- MAGE3-mIgGFc.

Example 7 Expression of fusion proteins

Expression of fusion proteins in CHO cells: Plasmids generated in the above Examples were transfected into CHO cells using Superfect (Qiagen) transfection reagent. Selection of transfected cells was performed with culture in Zeocin. Fusion protein secretion from CHO cells was measured by an mIgG ELISA. To enable high concentration of secretion, cells were subcloned in microtiter plates and tested for high level producers by ELISA. Using a high density culture system (Technomouse from Technomara) concentrations of secreted fusion proteins ranging from 1µg/ml to 15µg/ml were achieved. The supernatants of transfected cells were collected and stored at -80°C until usage.

Expression of SCP1 antigen dimers in *Pichia pastoris*: The cDNA-IgG insert in pSecTagA-SCP1A-mIgGFc was released from vector DNA by restriction digestion with *Sfi1/Sal1*. The released DNA (SCP1-mIgGFc-myc) was ligated into pPicZalphaB vector (Invitrogen) precut with *Sfi1/Sal1*. The resulting vector, pPicZalphaB-SCP1A-mIgGFc, allows the production of secreted proteins in *Pichia pastoris* containing a C-terminal myc and His6 tail. The generated plasmid was electroporated into *Pichia pastoris* and used for expression. Expression of secreted fusion proteins in supernatants was determined by an IgG ELISA with up to 10µg/ml fusion protein under standard non optimized culture conditions. Using high density fermentation 100-fold higher concentrations are expected.

Example 8 Detection of antigen specific B lymphocytes in tumor specimens.

For the detection of antigen specific *in situ* B cells and plasma cells cryosections of human tumors were prepared. These sections were stained with the fusion proteins in 1µg/ml concentration (a prior enrichment or purification of the fusion protein (SEREX-Ag-Dimers)) is not necessary. *In situ* bound SEREX-Ag dimers were visualized with secondary IgG specific antibodies linked to enyzmes or biotin using standard systems. Staining of B-cells and plasma cells were observed in breast cancer tissues for SCP1-Fc and NY-Eso1-Fc. The nature of stained cells was analyzed by staining of parallel tissue

sections with CD20 or anti-human-IgG antibodies. 60 tumor samples were tested. Nearly one third of tested tumors express significant amounts of SCP1-Ag binding B lymphocytes (the number may be underestimated since several tumor specimens were very small). Some tumors have a high density of antigen specific B-lymphocytes. In the majority of cases there are focal aggregates of antigen specific B lymphocytes. Most antigen specific cells are located in fibrous areas. In several cases single positive cells are scattered through the tumor sample.

It should be understood that the foregoing discussion and examples merely present a detailed description of certain preferred embodiments. It therefore should be apparent to those of ordinary skill in the art that various modifications and equivalents can be made without departing from the spirit and scope of the invention. All articles, patents and patent applications that are identified above are incorporated by reference in their entirety.

Claims

1. A method of generating recombinant human antibodies or antibody fragments directed against a cancer associated tumor antigen comprising the steps of:

a) isolating peripheral blood lymphocytes from tumor bearing patients;

b) detecting B-cells producing antibodies from said lymphocytes;

c) isolating cDNA encoding immunoglobulin from the B-cells;

d) expressing the immunoglobulin encoded by the cDNA of step c); and

e) isolating a functional antibody or antibody fragment, thereby generating a human antibody or antibody fragment against the cancer associated tumor antigen.

2. The method of claim 1, wherein said detection step comprises the binding of a cancer associated tumor antigen-fusion protein to the B-cells.

3. The method of claim 2, wherein said fusion protein comprises an immunoglobulin Fc constant region.

4. The method of claim 3, wherein said cancer associated tumor antigen is selected from the group consisting of SCP-1, NY-ESO-1, tyrosinase, cErbB2, MAGE-3 and HERV-K10.

5. The method of claim 1, wherein the step of isolating cDNA comprises:

a) extracting RNA from antibody producing B-cells;

b) synthesizing a double stranded cDNA pool; and

c) amplifying immunoglobulin cDNA from the double stranded cDNA pool by PCR.

6. The method of claim 1, wherein the step of expressing the immunoglobulin comprises:

a) ligating the isolated immunoglobulin encoding cDNA into an expression vector;

b) introducing said expression vector into host cells to express the immunoglobulin encoded by the ligated cDNA; and

c) growing cells under conditions wherein the encoded immunoglobulin is expressed by the host cells.

7. The method of claim 6, wherein the step of introducing the expression vector into host cells is effected by a process selected from the group consisting of transfection, infection and transformation.

8. The method of claim 6, wherein the host cells are prokaryotic or eukaryotic cells.

9. The method of claim 8, wherein the host cells are eukaryotic.

10. The method of claim 9, wherein the eukaryotic cells are selected from the group consisting of yeast cells, CHO cells, and insect cells.

11. The method of claim 9, wherein the expressed immunoglobulin is secreted into the medium.

12. The method of claim 8, wherein the host cells are prokaryotic cells.

13. The method of claim 12, wherein the prokaryotic cells are infected with a filamentous bacteriophage expression vector.

14. The method of claim 13, wherein the expressed immunoglobulin is displayed on the surface of the filamentous phage.

15. The method of claim 1, wherein the isolation of functional antibody comprises the steps of:

a) separating the expressed immunoglobulin from the host cells; and

b) purifying the separated immunoglobulin by chromatography.

16. The method of claim 15, wherein the chromatography process is selected from the group consisting of Zn-chelating sepharose, protein-A sepharose and affinity chromatography.

17. The method of claim 16, wherein the chromatography is affinity chromatography and wherein the stationary phase ligand comprises an antigen-Fc chimera which binds to the B-cells.

18. The method of claim 17, wherein said Fc chimera comprises a cancer associated tumor antigen and an immunoglobulin Fc constant region.

19. The method of claim 18, wherein said cancer associated tumor antigen is selected from the group consisting of SCP-1, NY-ESO-1, tyrosinase, cErbB2, MAGE-3 and HERV-K10.

20. A recombinant antibody isolated by the method any one of the claims 1-19.

21. A method of purifying human B-cells which express antibody which specifically binds to cancer associated tumor antigens, comprising the steps of:

a) binding an antigen-Fc chimera to the B-cells;

b) binding a fluorescent labeled antibody directed against the Fc component of the chimera; and

c) separating the labeled cells by automated cell sorting.

22. The method of claim 21, wherein the separation of labeled cells is carried out by FACS.

23. The method of claim 21, wherein said detection comprises the binding of an antigen-Fc chimera to the B-cells.

24. The method of claim 23, wherein the antigen-Fc chimera comprises a cancer associated tumor antigen and an immunoglobulin Fc constant region.

25. The method of claim 24, wherein said cancer associated tumor antigen is selected from the group consisting of SCP-1, NY-ESO-1, cErbB2, MAGE-3 and HERV-K10.

26. An antigen-Fc chimera which comprises a cancer associated tumor antigen at the N-terminus and an immunoglobulin Fc constant region at the C-terminus.

27. The antigen-Fc chimera of claim 26, wherein the immunoglobulin Fc constant region is a murine constant region fragment from IgG1.

28. The antigen-Fc chimera of claim 27, wherein said cancer associated tumor antigen is selected from the group consisting of SCP-1, NY-ESO-1, tyrosinase, cErbB2, MAGE-3 and HERV-K10.

29. An antigen-Fc chimera comprising SCP-1 at the N-terminus and a murine constant region fragment from IgG1 at the C-terminus.

30. An antigen-Fc chimera comprising NY-ESO-1 at the N-terminus and a murine constant region fragment from IgG1 at the C-terminus.

31. An antigen-Fc chimera comprising cErbB2 at the N-terminus and a murine constant region fragment from IgG1 at the C-terminus.

32. An antigen-Fc chimera comprising MAGE-3 at the N-terminus and a murine constant region fragment from IgG1 at the C-terminus.

33. An antigen-Fc chimera comprising HERV-K10 at the N-terminus and a murine constant region fragment from IgG1 at the C-terminus.

34. A nucleic acid encoding an antigen-Fc chimera which comprises a cancer associated tumor antigen at the N-terminus and an immunoglobulin Fc constant region at the C-terminus.

35. The nucleic acid of claim 34, wherein said cancer associated tumor antigen is selected from the group consisting of SCP-1, NY-ESO-1, tyrosinase, cErbB2, MAGE-3 and HERV-K10.

36. A nucleic acid encoding an antigen-Fc chimera comprising SCP-1 at the N-terminus and a murine constant region fragment from IgG1 at the C-terminus.

37. A nucleic acid encoding an antigen-Fc chimera comprising NY-ESO-1 at the N-terminus and a murine constant region fragment from IgG1 at the C-terminus.

38. A nucleic acid encoding an antigen-Fc chimera comprising cErbB2 at the N-terminus and a murine constant region fragment from IgG1 at the C-terminus.

39. A nucleic acid encoding an antigen-Fc chimera comprising MAGE-3 at the N-terminus and a murine constant region fragment from IgG1 at the C-terminus.

40. A nucleic acid encoding an antigen-Fc chimera comprising HERV-K10 at the N-terminus and a murine constant region fragment from IgG1 at the C-terminus.

41. A method of detecting B cells which produce tumor antigen specific antibodies in a sample, comprising the steps of:

a) exposing the sample to a fusion protein comprising the tumor antigen; and

b) determining whether the fusion protein bound to the B cells.

INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/US 99/22314

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K16/00 G01N33/68 C12N15/13 C07K16/30 C07K16/32
C12N5/08 C12N15/62 C07K19/00 C07K14/82 //C12N15/63,
C07K1/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YIP Y ET AL: "Evaluation of different lymphoid tissue sources for the construction of human immunoglobulin gene libraries." IMMUNOTECHNOLOY, vol. 3, 1997, pages 195-203, XP004097003 page 201, right-hand column, line 27 -page 202, right-hand column, line 18 abstract	1
A	STOCKERT E ET AL: "A survey of the humoral immune response of cancer patients to a panel of human tumor antigens 'see comments!." JOURNAL OF EXPERIMENTAL MEDICINE, (1998 APR 20) 187 (8) 1349-54. , XP000867818 page 1352, left-hand column, line 11 -page 1353, right-hand column, line 44	1-41

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 February 2000	17/02/2000

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 99/22314

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94 11507 A (GINGERAS T) 26 May 1994 (1994-05-26) claims 1-4 ---	1-41
A	TURECI O ET AL: "Identification of a meiosis-specific protein as a member of the class of cancer/testis antigens." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1998 APR 28) 95 (9) 5211-6. , XP002129691 abstract ---	4,25-40
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Information on patent family members

Inte onal Application No
PCT/US 99/22314

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		EP 0628076 A	14-12-1994